Multimodal Microscopy for Tissue Diagnostics

Label-Free, Noninvasive, High-Resolution and Molecule Specific

Multimodal nonlinear microscopy using near infrared excitation lasers enables noninvasive, label-free high resolution imaging of native tissue. The methods are highly promising for biomedical applications, e.g., frozen section analysis in histopathology, in vivo intraoperative imaging and endoscopy, since disease induced morphologic and chemical changes can be directly visualized at subcellular spatial resolution without any need for contrast agents, when combined with novel laser technology.

Multimodal nonlinear microscopy combining coherent anti-Stokes Raman scattering (CARS), two-photon excited fluorescence (TPEF) and second harmonic generation (SHG) using near infrared (NIR) excitation lasers provides significant advantages in comparison to nowadays established biomedical imaging methods [1]. First, the nonlinear intensity dependence confines the signal generation to the laser focus of highest intensity, which results in intrinsic 3D-sectioning capability and higher signal detection efficiency in particular deep within tissue, since a confocal detection system collecting ballistic photons only is not needed. NIR excitation increases the penetration depth further, such that more than 1 mm of penetration depth has been realized so far for three photon fluorescence [2]. Furthermore, CARS and autofluorescence of endogenous molecular markers are label-free, such that the methods can be directly applied to native tissue without need for labelling ideally suited for in vivo application. Thus, one prominent field of application is intraoperative histopathology. Within the last years we could show, that the combination of CARS, TPEF and SHG is a powerful approach for label free clinical tissue diagnostics [3,4,5,6].

Material & Methods

Multimodal nonlinear imaging experiments were performed using a Ti:Sa-laser (Coherent, USA). The output of this laser at 830 nm is divided using a non-polarizing beam splitter. The first fraction pumps an optical parametric oscillator (APE, Germany) generating a continuously tunable laser of 300 mW output power in the range of 500 to 800 nm, which is used as CARS pump beam.
The other part of the Ti:Sa-laser is used as excitation laser for SHG and TPEF imaging and as Stokes beam for CARS imaging. Pump and Stokes are spatially overlapped and temporally synchronized using a dichroic mirror and a mechanical delay line and fed into a laser-scanning microscope (Zeiss, Germany). For CARS microscopy experiments at the aliphatic CH-stretching vibration of methylene groups CH2 at 2850 cm\(^{-1}\), the pump beam is tuned to 670 nm. CARS and SHG signals are both primarily emitted in forward direction and collected by a condenser, while the TPEF signal is detected in backward direction through the microscope objective. The signals are spectrally separated by dichroic beam splitters, short and band-pass filters and detected by photomultiplier tubes. The setup is displayed in figure 1a). Further miniaturization for clinical application can be realized by using compact fiber lasers as pump sources, which are well suited for multimodal nonlinear microscopy (fig. 1b) [7]. Using these lasers, compact devices for clinical application can be produced (fig. 1c) [8]. Thin frozen tissue sections of 20 µm thickness were used without further processing.

**Results & Discussion**

Figure 2 shows as an example a CARS/TPEF/SHG image of a thin skar tissue of human skin section, where the CARS signal is coded in red, TPEF in green and SHG in blue. CARS at 2850 cm\(^{-1}\) highlights in particular the distribution of lipids and macromolecules of the biological matrix in general, while SHG is sensitive to non-centrosymmetric molecules, in particular quasi-crystalline protein fibers made of collagen. TPEF is used to investigate the spatial distribution of autofluorescing molecules, which are in particular cofactors like NAD(P)H, FAD and dyes like melanin, but also elastic fibers. The combination of these three imaging modalities enables both the visualization of the tissue morphology and its chemical composition. Here, the skar tissue is characterized by relatively strong SHG signals, as evident in figure 2 and studying its structure provides information on the status of the healing process. As shown in figure 1c), the equipment needed to perform
such measurements can be readily implemented into compact devices for a wide range of clinical applications, e.g., for the detection of skin cancer [3] or intraoperative frozen section analysis for the detection of the tumor margin of head and neck carcinoma [4]. Here it was found, that cancer formation within the epithelial layer is accompanied by reduced lipid content and an increased metabolic activity, which can be detected by reduced CARS signal levels and increasing fluorescence signal levels. The hallmark of invasive cancer growth, e.g., breaking of cancer cells through the basal cell layer, can be visualized by SHG imaging of the integrity of the collagen matrix. In particular in combination with multivariate statistical data analysis, multimodal nonlinear imaging can be used to generate histopathologic staining equivalent images or to automatically detect the disease status of the tissue sample under investigation, e.g. for assessing disease activity in order to diagnose inflammatory bowel diseases (IBD) and discriminate Crohn’s disease from ulcerative colitis [5, 6].

While these results focus on a rather simple experimental setting using a single combination of pump and Stokes laser for excitation to extract simultaneously SHG, TPEF and CARS signals at 2850 cm$^{-1}$, even very subtle changes may be visualized by hyperspectral CARS imaging using tunable fiber lasers [9, 10]. For example imaging at 2850 cm$^{-1}$ and 2930 cm$^{-1}$ enables discriminating lipid from protein distribution [11]. The existing tuning concepts of compact fiber lasers cover already a significant part of the Raman spectrum from ~900 to 3500 cm$^{-1}$ for investigating the spatial distribution of virtually any molecular marker of sufficiently high concentration.

While one great advantage of multimodal nonlinear imaging is that labels are not required, the concept is also compatible with all established labeling techniques. TPEF can be used to detect virtually any fluorescent marker. Here, the two-photon excitation cross sections are typically much broader, such that tuning of the excitation laser is not that critical. Alternatively to fluorescence, recently Raman tags based on isotope labelling and alkyne tagging have been developed, which can be used for simultaneous tracking of up to 24 marker molecules due to the great multiplexing capabilities of Raman scattering in comparison to fluorescence, since the spectral line width is much smaller [12].

If additional information on various length scales is needed to fully characterize the sample of interest, which is particularly relevant for in vivo imaging, multimodal nonlinear imaging can be further combined with a wide range of other nonlinear or linear imaging modalities. Three-photon excited fluorescence and third harmonic generation microscopy are based on lasers at about 1.5 µm and provide larger penetration depths [2]. Two-photon fluorescence lifetime imaging enables
differentiation of free from NAD(P)H bound protein, which have identical emission spectra but the fluorescence lifetime differs by one order of magnitude. Optical coherence tomography can be used to measure depth profiles of tissue samples, while hyperspectral imaging enables assessing the oxygenation status and water content of tissue, hence the set of methods can be tailored to meet the requirements of specific biomedical imaging applications [13].

Summary & Conclusion

Multimodal nonlinear microscopy using CARS, TPEF and SHG can be used to investigate the structure and composition of complex tissues and is as such well suited to detect subtle changes in morphology and the distribution of endogenous molecular markers connected to disease initiation and disease progression in a label free and noninvasive manner. It was already shown, that this set of methods can be successfully implemented into endoscopic probes ready for in vivo imaging applications [14, 15]. In combination with online data analysis, multimodal nonlinear microscopy is a powerful tool supporting established methods in digital histopathology but also enables addressing currently unmet biomedical needs, e.g., precise intraoperative recognition of tumor margins or the detection of single tumor cells. In combination with future developments in laser and detection technology, robust and compact devices for application in clinical routine diagnostics can be realized.

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